THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 283, NO. 24, pp. 16711–16722, June 13, 2008

Mitotic Phosphorylation Stimulates DNA Relaxation Activity of Human Topoisomerase I*[S]

Received for publication, September 14, 2007, and in revised form, March 20, 2008 Published, JBC Papers in Press, April 11, 2008, DOI 10.1074/jbc.M802246200

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Human DNA topoisomerase I (topo I) is an essential mammalian enzyme that regulates DNA supercoiling during transcription and replication. In addition, topo I is specifically targeted by the anticancer compound camptothecin and its derivatives. Previous studies have indicated that topo I is a phosphoprotein and that phosphorylation stimulates its DNA relaxation activity. The locations of most topo I phosphorylation sites have not been identified, preventing a more detailed examination of this modification. To address this issue, mass spectrometry was used to identify four topo I residues that are phosphorylated in intact cells: Ser[10], Ser[21], Ser[112], and Ser[394]. Immunoblotting using anti-phosphoepitope antibodies demonstrated that these sites are phosphorylated during mitosis. *In vitro* kinase assays demonstrated that Ser[10] can be phosphorylated by casein kinase II, Ser[21] can be phosphorylated by protein kinase Cα, and Ser[112] and Ser[394] can be phosphorylated by Cdk1. When wild type topo I was pulled down from mitotic cells and dephosphorylated with alkaline phosphatase, topo I activity decreased 2-fold. Likewise, topo I polypeptide with all four phosphorylation sites mutated to alanine exhibited 2-fold lower DNA relaxation activity than wild type topo I after isolation from mitotic cells. Further mutational analysis demonstrated that Ser[21] phosphorylation was responsible for this change. Consistent with these results, wild type topo I (but not S21A topo I) exhibited increased sensitivity to camptothecin-induced trapping on DNA during mitosis. Collectively these results indicate that topo I is phosphorylated during mitosis at multiple sites, one of which enhances DNA relaxation activity *in vitro* and interaction with DNA in cells.

Human topo I[2] is a type IB topoisomerase that relieves positive and negative DNA supercoiling caused by transcription, replication, and chromosome condensation (1). The 91-kDa, 765-amino acid polypeptide contains four domains: a poorly conserved lysine-rich N-terminal domain that contains nuclear and nucleolar localization signals, a linker region, and the core and C-terminal domains that contain the residues important for DNA interaction and relaxation of supercoils (2). A transesterification reaction at the active site of topo I ligates Tyr[723] of the enzyme to the 3′ phosphate of the DNA, thereby creating a nick in the DNA backbone (3). This nick allows controlled rotation of the DNA to relieve supercoils. Mutation of Tyr[723] prevents the transesterification reaction and abolishes all relaxation activity (4). The anticancer drug CPT and its derivatives slow topo I-mediated DNA relaxation (5, 6) and inhibit the religation reaction step of the enzyme (7, 8), trapping topo I on DNA (9, 10) and causing cell death (11, 12).

A number of observations have raised the possibility that phosphorylation can modulate the activity and CPT sensitivity of topo I. Treatment with calf intestine alkaline phosphatase decreases topo I enzymatic activity *in vitro* (13–15). Conversely subsequent treatment with PKC or CKII, two kinases that copurify with topo I and phosphorylate it *in vitro* (16–18), stimulates topo I activity 2–3-fold (14, 15, 19) and enhances the ability of CPT to trap covalent topo I-DNA cleavage complexes (20), suggesting that phosphorylation by these kinases might make topo I more sensitive to CPT.

Despite its potential importance, many aspects of topo I phosphorylation remain poorly understood. The number of phosphorylation sites, for example, remains unclear because the number of phosphopeptides detected after metabolic labeling and immunoprecipitation has varied from one (16) to as many as five (21) or six (17). The effect of cell cycle progression on phosphorylation has likewise been unclear. Initial studies suggested that topo I is phosphorylated in interphase cells as evidenced by its labeling in unsynchronized cell populations and a rapid increase in phosphorylation after certain treatments (13, 16, 17, 20, 21). In contrast, a more recent study found that topo I in rodent cells quantitatively shifts to a slower migrating, phosphorylated state exclusively during mitosis (22). Finally the location of topo I phosphorylation sites has not been resolved. Cardellini *et al.* (23) reported that a 17-amino acid peptide from the N terminus of topo I could be phosphorylated at Ser[10] by CKII *in vitro*, but phosphorylation of this site was not verified in the full-length polypeptide *in vitro* or *in vivo*. More recently, Yu *et al.* (24) reported that topo I is phosphorylated on Tyr[268] by c-Abl *in vitro*, but again the phosphorylation of this site in intact cells was not confirmed. The location of other possible phosphorylation sites on topo I, the cellular conditions

* This work was supported, in whole or in part, by National Institutes of Health Grant R01 CA73709. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S4.

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[2] The abbreviations used are: topo, topoisomerase; Cdk, cyclin-dependent kinase; CKII, casein kinase II; CPT, camptothecin; PBS, calcium- and magnesium-free Dulbecco's phosphate-buffered saline; PKC, protein kinase C; PMSF, α-phenylmethylsulfonyl fluoride.

that cause phosphorylation, and the effects of individual phosphorylation events on topo I remain largely unknown.

In the present study, we utilized mass spectrometry to map four phosphorylation sites on topo I, generated phosphoepitope-specific antibodies, and used these antibodies to study the conditions leading to topo I phosphorylation *in vitro* and in intact cells. In addition, we performed site-directed mutagenesis of these sites to assess the impact on localization and activity. Results of this analysis suggest that topo I is phosphorylated during mitosis in untreated cells and that one of these mitotic phosphorylations modestly enhances topo I activity *in vitro* as well as sensitivity to CPT-induced trapping on DNA in intact cells.

EXPERIMENTAL PROCEDURES

Materials—Reagents were obtained from the following suppliers: CPT, paclitaxel, aphidicolin, hydroxyurea, Hoechst 33258, PMSF, phorbol myristate acetate, and chloroquine from Sigma; nocodazole from Aldrich; and DMSO from Fisher. Antibodies to the following antigens were obtained from the indicated suppliers: nucleolin from Santa Cruz Biotechnology, Inc., TATA-binding protein from BD Transduction Laboratories, and topors from Novus. Anti-S peptide antibody was raised and characterized as described previously (25). Antibodies to other antigens were kind gifts from the following investigators: murine monoclonal anti-poly(ADP-ribose) polymerase from Guy Poirier (Laval University, Ste. Foy, Quebec, Canada), human anti-topo I autoantiserum from Naomi Rothfield (University of Connecticut, Farmington, CT), and C21 murine monoclonal anti-topo I from Y.-C. Cheng (Yale University Medical School, New Haven, CT).

Cell Culture—A549 human lung cancer cells and K562 human leukemia cells were grown in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin G, 100 μg/ml streptomycin, 2 mM L-glutamine (medium A). Cells were incubated at 37 °C in a humidified incubator with 5% CO_2.

In the indicated experiments, cells were treated with 100 nM paclitaxel for 16 h, resulting in arrest of >80% of cells in G_2/M as assessed by flow cytometry and 75–80% of cells in metaphase as assessed by fluorescence microscopy after Hoechst 33258 staining. Longer exposures to paclitaxel were avoided because of adaptation of the mitotic checkpoint and exit of these cells into a multinucleated, tetraploid G_1 state (26).

Plasmid Construction—The yeast shuttle vector hGal1 containing cDNA encoding catalytically inactive (Y723F) human topo I was a kind gift from M.-A. Bjornsti (St. Jude Children's Hospital, Memphis, TN). The cDNA was subjected to PCR to add the S peptide and linker sequence (in brackets) [GAGAGAGAGGAP]MKETAAAKFERQHMDS or MKETAAAKFERQHMDS[GAGAGAGAGGAP] to either the C-terminal (Topo I-S) or N-terminal (S-Topo I) end of the topo I open reading frame, respectively. After ligation of Topo I-S or S-Topo I into pcDNA 3.1 (Invitrogen), the entire insert was sequenced. Topo I-S was used for generating a stably transfected K562 cell line, which was then used for mass spectrometry and *in vitro* kinase assays as described below. S-Topo I was used for all other experiments.

Transient and Stable Transfection—10–15 $\times$ 10^6 K562 cells were washed in sterile PBS and resuspended in cytomix buffer (120 mM KCl, 150 μM $CaCl_2$, 2 mM EGTA, 5 mM $MgCl_2$, 25 mM HEPES, 10 mM K_2HPO_4/KH_2PO_4 (pH 7.6)) containing 40 μg of plasmid. Cells were electroporated at 320 V for 10 ms in a BTX ECM 830 square wave electroporator, incubated for 15 min at room temperature, and diluted in medium A without antibiotics. After 24 h, cells were utilized for the assays described below. Alternatively 48 h after electroporation, cells transfected with Topo I-S (Y723F) were treated with 800 μg/ml Geneticin to select stable transfectants. Once Geneticin-resistant cells grew, clones were isolated by limiting dilution and assayed for Topo I-S expression by immunoblotting using anti-S peptide antibody. A resulting stable line with high expression was maintained in medium A containing 400 μg/ml Geneticin.

Immunoprecipitation and Pulldown—Endogenous topo I was isolated from K562 cells using human anti-topo I autoantibody and protein A-Sepharose beads. All steps were performed at 4 °C. Cells were washed twice in ice-cold PBS, incubated in lysis buffer with protease and phosphatase inhibitors (1% (w/v) Triton X-100, 400 mM NaCl, 50 mM HEPES (pH 7.5), 10% (w/v) glycerol, 5 mM $MgSO_4$, 1 mM EDTA, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 100 mM NaF, 1 mM PMSF, 10 μg/ml leupeptin, 100 units/ml Trasylol, 1% (w/v) thiodiglycol, 20 nM microcystin) for 15 min, and sedimented at 12,000 $\times$ *g* for 15 min. Topo I antibody was added to the resulting supernatant, which was rotated end over end overnight. Samples were supplemented with protein A beads and rotated for an additional 4 h. Beads were spun down at 12,000 $\times$ *g* for 1 min and washed four times with RIPA buffer containing phosphatase inhibitors (1% (w/v) Triton X-100, 1% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 150 mM NaCl, 10 mM sodium phosphate (pH 7.2), 2 mM EDTA, 1 mM sodium orthovanadate, 100 units/ml Trasylol, 50 mM NaF). To elute protein for SDS-PAGE, beads were resuspended in sample buffer (4 M urea, 2% (w/v) SDS, 62.5 mM Tris-HCl (pH 6.8), 1 mM EDTA, 5% (v/v) 2-mercaptoethanol, 0.1% (w/v) bromphenol blue) and heated to 65 °C for 20 min.

S-Topo I or Topo I-S was isolated using a similar procedure. In brief, lysates were prepared as described above and sedimented at 12,000 $\times$ *g* for 15 min. S protein beads (Novagen) were added to the resulting supernatant, which was rotated end over end overnight. Beads were then sedimented and washed four times with RIPA buffer containing phosphatase inhibitors prior to SDS-PAGE or incubation with kinases. Alternatively immobilized topo I was washed with 0.1% Nonidet P-40 in PBS rather than RIPA buffer and assayed for topo I activity.

Metabolic Labeling—2.5 $\times$ 10^7 K562 cells were washed once and resuspended in 10 ml of phosphate- and serum-free RPMI 1640 medium (BIOSOURCE). After cells were incubated for 30 min at 37 °C, 2.5 mCi of [^{32}P]orthophosphate (Amersham Biosciences) and dialyzed fetal calf serum (10% (v/v) final concentration) were added for 4 h. Topo I was recovered from radiolabeled cells by immunoprecipitation or pulldown with S protein-agarose as described above, subjected to SDS-PAGE, and visualized by autoradiography.

Two-dimensional Tryptic Mapping—5.0 $\times$ 10^7 K562 cells stably transfected with Topo I-S (Y723F) were treated for

16 h with 100 nM paclitaxel and then radiolabeled with [^{32}P]orthophosphate as described above. Topo I pulled down with S protein-agarose was identified by SDS-PAGE followed by autoradiography. The radiolabeled topo I band was excised and subjected to reduction, alkylation, and digestion with 2 μg of trypsin as described by Meisenhelder *et al.* (27). The resulting sample was spotted onto a 20 × 20-cm, 100-μm cellulose thin layer chromatography plate (EM Science), subjected to electrophoresis at 1000 V for 30 min in pH 1.9 buffer, and exposed to ascending chromatography overnight in buffer consisting of 37.5% (v/v) *n*-butyl alcohol, 25% (v/v) pyridine, 7.5% (v/v) acetic acid. Radiolabeled spots were detected using a Storm 840 PhosphorImager (GE Healthcare).

Mass Spectrometry—Topo I-S was isolated from 1 × 10^7 stably transfected K562 cells, subjected to SDS-PAGE, and stained with Coomassie Blue. The topo I band was excised, digested using either trypsin or Arg-C, and analyzed by quadrupole time-of-flight tandem mass spectrometry at the Taplin Biological Mass Spectrometry Facility (Harvard Medical School, Boston, MA).

Generation of Phosphospecific Antibodies—Phosphorylated and nonphosphorylated peptides surrounding Ser^{10} (CDHLHND<u>S</u>QIEADFR), Ser^{21} (CADFRLND<u>S</u>HKHKDKH), Ser^{112} (CEKENGFS<u>S</u>PPQIKDE), and Ser^{394} (CSKDAKVP<u>S</u>PPPGHKW) were synthesized. Phosphorylated peptides were conjugated to keyhole limpet hemocyanin through their N-terminal cysteines and utilized to immunize rabbits. Bleeds were screened by immunoblotting using whole cell lysates of K562 cells and dot blots of both the phosphorylated and nonphosphorylated peptides coupled to bovine serum albumin at concentrations from 1 μg to 100 pg. Bleeds showing evidence of specificity for phosphorylated peptides were affinity-purified by sequential passage over SulfoLink columns (Pierce) derivatized with nonphosphorylated and phosphorylated peptides. Antibodies were eluted in 100 mM glycine (pH 2.5), neutralized with 1 M Tris-HCl (pH 9.5), assayed by dot blot and whole cell lysate immunoblotting, diluted 1:1 in glycerol, and stored at −20 °C. The specificity of the antibodies was tested by a peptide competition assay in which blots of mitotic K562 lysates were probed with each phosphospecific antibody in the absence or presence of 1 μg/ml synthetic phosphopeptide as indicated.

Immunoblotting—Western blotting of whole cell lysates, immunoprecipitates, or pulldown samples was performed on nitrocellulose membranes as described previously (28). Either ECL (Amersham Biosciences) or SuperSignal (Pierce) enhanced chemiluminescence reagents were used depending on the strength of the antibody.

Cell Cycle Analysis of Topo I Phosphorylation—K562 cells were treated for 16 h with 10 μM aphidicolin, 2 mM hydroxyurea, 100 nM paclitaxel, 150 nM nocodazole, or diluent (0.1% DMSO). At the end of the incubation, cells were harvested, subjected to SDS-PAGE, and analyzed by immunoblotting using phosphoepitope-specific anti-topo I antibodies. Duplicate aliquots were fixed in 50% ethanol, treated with RNase A, stained with propidium iodide, and subjected to flow cytometry as described previously (29).

To isolate mitotic cells without the use of pharmacological agents, mitotic shake-off (30) was performed. T175 flasks of log phase A549 cells were vigorously agitated for l min. After the loosened cells and medium were removed, fresh medium was added to the flasks. The process was repeated every 30 min for 9 h. The initial aliquot of dislodged cells was discarded. Thereafter cells were sedimented at 100 × *g*, resuspended in ice-cold RPMI 1640 medium, 10 mM HEPES (pH 7.4), pooled, and stored on ice. The isolated mitotic cells and remaining control adherent cells were stained with Hoechst 33258 to determine mitotic index and were subjected to immunoblotting.

In Vitro Kinase Assay—Topo I-S pulled down from stably transfected K562 cells using S protein beads or commercially available purified topo I (Topogen, Columbus, OH) was mixed with 200 μM ATP and 10 units of purified CKII or Cdk1 (New England Biolabs) in the CKII or Cdk1 reaction buffers provided by the supplier. Alternatively the beads were incubated with PKC reaction buffer (20 mM HEPES-NaOH (pH 7.4), 0.03% Triton X-100) containing lipid activator (100 μg/ml phosphatidylserine, 200 pM phorbol myristate acetate, 0.03% Triton X-100, 100 μM dithiothreitol), 100 μM $CaCl_2$, 200 μM ATP, 1 mM magnesium acetate, and 10 ng of PKCα (Upstate) in a 30 °C water bath for 30 min. After SDS sample buffer was added, samples were heated to 65 °C for 15 min, subjected to SDS-PAGE, and analyzed by immunoblotting.

Site-directed Mutagenesis—Mutations were introduced by site-directed mutagenesis using a QuikChange^{TM} mutagenesis kit (Stratagene) according to the instructions of the supplier. The complete sequences of the topo I open reading frames were verified by sequencing after every mutagenesis. S-Topo I was mutated to restore the enzyme active site (Tyr^{723}) and to create phosphorylation site mutants, including the quadruple phosphomutant "4A" (S10A/S21A/S112A/S394A), the triple mutant "3A" (S10A/S112A/S394A), and the S21A mutant.

Immunofluorescence—K562 cells were transiently transfected with plasmids encoding wild type or phosphomutant active (Tyr^{723}) S-Topo I. 24 h later, cells were washed in PBS and spun onto glass slides at 60 × *g* for 5 min using a Shandon cytocentrifuge. Cells were fixed in ice-cold methanol for 10 min, rehydrated in PBS, and blocked in TSM (150 mM NaCl, 10 mM Tris-HCl (pH 7.4) containing 10% (w/v) nonfat milk powder, 100 units/ml penicillin G, 100 μg/ml streptomycin, 1 mM sodium azide) at 20–22 °C for 1 h. After anti-S peptide antibody was added (10 μg/ml in TSM), samples were incubated at 4 °C overnight. Slides were washed six times with PBS and treated with fluorescein isothiocyanate-conjugated anti-mouse IgG secondary antibody (20 μg/ml in TSM; Kirkegaard & Perry Laboratories, Inc. (KPL), Gaithersburg, MD) for 45 min at 37 °C. Slides were then washed six times with PBS and treated with 1 μg/ml Hoechst 33258 to locate nuclei and chromosomes. Cells were visualized using a Zeiss LSM 510 confocal microscope.

Binding Partner Analysis—Plasmids encoding wild type or 4A S-Topo I were transfected into K562 cells. Beginning 6 h after transfection, 100 nM paclitaxel was added to cells for 18 h to induce mitotic arrest. After incubation, topo I was recovered with S protein beads, subjected to SDS-PAGE, and analyzed by

immunoblotting with antibodies against various topo I binding partners.

Topo I Activity Assays—Plasmids encoding wild type or phosphorylation site mutant S-Topo I were transfected into K562 cells. Beginning 6 h after transfection, 100 nM paclitaxel was added to cells to induce a mitotic arrest. After an 18-h incubation in paclitaxel, topo I was recovered with S protein-agarose. Beads were washed four times in 0.1% Nonidet P-40 in PBS because RIPA buffer was found to abolish topo I activity. A fraction of the beads was removed for immunoblotting to quantify S-Topo I expression. Where indicated, 10 units of purified calf intestine alkaline phosphatase (Invitrogen) was added, and beads were incubated at 37 °C for 30 min. Plasmid relaxation activity of the immobilized topo I was then analyzed as described by Hann *et al.* (31). In brief, the beads were diluted in 2-fold increments and resuspended in topo I activity buffer (50 mM Tris-HCl (pH 7.5), 2.5 mM $MgCl_2$, 110 mM NaCl, 500 μM EDTA, 6 μg/ml bovine serum albumin, 0.01% (w/v) dithiothreitol).

Alternatively, after treatment of K562 cells with 0.1% DMSO or 100 nM paclitaxel for 16 h, cells were sedimented, washed once in PBS, and resuspended in nuclear isolation buffer consisting of 10 mM NaCl, 10 mM Tris-HCl (pH 7.4), 3 mM $MgSO_4$ containing 1 mM dithiothreitol, 100 units/ml Trasylol, 1 mM PMSF, 0.5 mM EDTA, 10 mM NaF, 2 mM $Na_2P_2O_7$, 1 mM Na_2VO_4, 20 nM microcystin. After a 20-min incubation on ice, samples were subjected to 30 strokes in a tight fitting Dounce homogenizer, examined microscopically to confirm cell disruption, and sedimented at 16,000 × *g* for 15 min to sediment nuclei and mitotic chromosomes, respectively. After a wash with nuclear isolation buffer, pellets were resuspended in 75 μl of topoisomerase extraction buffer consisting of 100 mM sodium phosphate (pH 7.4), 1 mM EDTA, 0.1 mM dithiothreitol, 1 mM PMSF, 10 mM NaF, 2 mM $Na_2P_2O_7$, 1 mM Na_2VO_4, 20 nM microcystin; treated with an equal volume of topoisomerase extraction buffer containing 1.2 M KCl; and incubated on ice for 15 min. After DNA was precipitated from the extracts using polyethylene glycol (32), supernatants were adjusted to equal protein concentrations. Aliquots recovered by precipitation in 20% (w/v) ice-cold trichloroacetic acid were subjected to SDS-PAGE and immunoblotting for topo I content. For end point assays, aliquots containing serial 2-fold dilutions of the extracts in topo I activity buffer supplemented with the phosphatase inhibitors 10 mM NaF, 1 mM $Na_2P_2O_7$, and 20 nM microcystin were assayed for ability to relax supercoiled plasmid. Reactions were initiated by adding 500 ng of $O6^{\#7}$ plasmid DNA and incubated at 37 °C for 30 min. After dilution with 1/10 volume containing 1 μg of proteinase K in 10% (w/v) SDS, samples were incubated at 37 °C for another 15–30 min. For time course experiments, reactions containing equal amounts of topo I as assessed by immunoblotting were incubated with 5 μg of plasmid $O6^{\#7}$ in 180 μl of topo I activity buffer with phosphatase inhibitors for 0–30 min. At the indicated times, 20-μl aliquots were treated with 1 μg of proteinase K and 1% SDS (final concentration) at 50 °C to stop the reaction. Plasmids were separated on 1% agarose gels in TPE buffer (36 mM Tris, 30 mM NaH_2PO_4, 1 mM EDTA, pH 7.8) containing 10 μg/ml chloroquine, stained with 500 ng/ml ethidium bromide, and visualized under 260 nm illumination.

Topo I Cleavage Half-reaction—Based on the results of Pourquier *et al.* (33), substrate was generated by annealing a 16-mer upper strand (5′-GATCTAAAAGACTTGG-3′) to a 36-mer bottom strand (5′-GATCTTTTTTAAAAATTTTTCCAAGTCTTTTACATC-3′). The annealed substrate was labeled with 800 Ci/mol [α-^{32}P]dATP (PerkinElmer Life Sciences) and 5 units of Klenow fragment (New England Biolabs) to add five radiolabeled adenosines to the upper strand. The radiolabeled duplex was separated from unincorporated nucleotide on a Sephadex TE-10 spin column.

After K562 cells transiently transfected with plasmid encoding wild type or S21A topo I were incubated with 100 nM paclitaxel for 16 h, topo I was pulled down using S protein-agarose as described above. An aliquot containing 40% of the beads was incubated with 300 fmol of radiolabeled substrate in topo I activity buffer at 37 °C. At the indicated times, aliquots containing 50 fmol of substrate were removed, diluted with formamide to a final concentration of 60%, heated to 100 °C for 10 min, cooled on ice, and separated on a denaturing 10% polyacrylamide gel (19:1 acrylamide:bisacrylamide, 7.5 M urea). Substrate incubated for 45 min at 37 °C in buffer lacking topo I and *bona fide* 7-mer (6-mer annealed to the bottom strand and extended with Klenow as described above) were included on each gel as markers. Gels were examined on a PhosphorImager as described above. The remainder of each pulldown was utilized for immunoblotting.

Band Depletion Assays—K562 cells transiently transfected with plasmids encoding wild type or S21A S-Topo I were treated with 0.1% DMSO or 100 nM paclitaxel for 16 h as described above. Alternatively untransfected K562 cells were treated with 0.1% DMSO or 100 nM paclitaxel for 16 h to examine endogenous topo I. Cells were sedimented at 100 × *g* for 10 min, resuspended in serum-free RPMI 1640 medium containing 10 mM HEPES (pH 7.4 at 21 °C), diluent or 100 nM paclitaxel, and diluent *versus* 1.5, 5, 15, or 50 μM CPT. After a 45-min incubation at 37 °C, cells were briefly sedimented at 9600 × *g*, resuspended in lysis buffer (6 M guanidine hydrochloride, 250 mM Tris-HCl (pH 8.5 at 21 °C), 10 mM EDTA, 1% (v/v) freshly added β-mercaptoethanol, 1 mM PMSF), and sonicated. Samples were alkylated with iodoacetamide, dialyzed, and lyophilized as described previously (34). After SDS-PAGE and immunoblotting, bands were quantified with ImageJ software.

RESULTS

Topo I Is Phosphorylated in Cells—When topo I was immunoprecipitated from [^{32}P]orthophosphate-labeled log phase K562 human leukemia cells using a human anti-topo I antiserum (35), subsequent SDS-PAGE and autoradiography indicated that the topo I polypeptide contained covalently bound ^{32}P (Fig. 1*A*) in agreement with previous results suggesting that topo I is phosphorylated in intact cells (13, 16, 17, 20–22). To permit isolation of the large amounts of purified topo I polypeptide required for mapping the phosphorylation sites, the S peptide epitope tag and a linker were inserted on either the C terminus (Topo I-S) or N terminus (S-Topo I) of the topo I open reading frame (25). Because of the potential toxicity associated

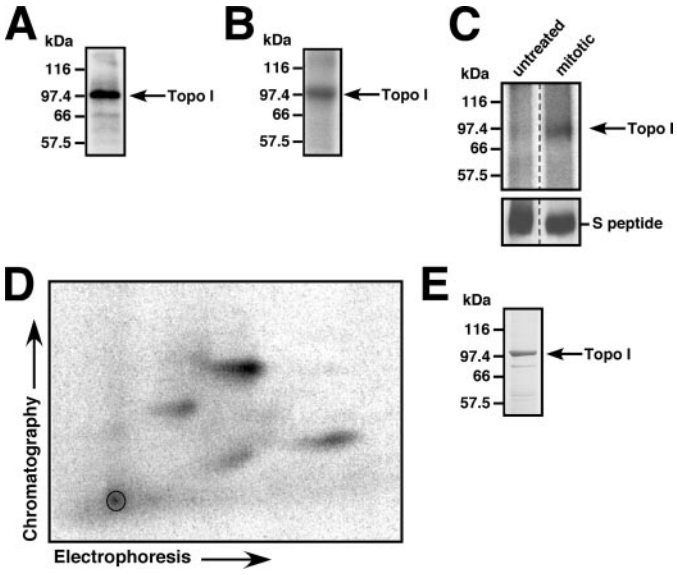

FIGURE 1. **Endogenous and S peptide-tagged topo I is phosphorylated in cells.** *A*, after 2.5×10^7 K562 human leukemia cells were radiolabeled for 4 h with 0.25 mCi/ml [^{32}P]orthophosphate, topo I was isolated by immunoprecipitation using human anti-topo I antiserum, subjected to SDS-PAGE, and analyzed by autoradiography. *B*, after 2.5×10^7 K562 cells stably expressing topo I-S (Y723F) were labeled for 4 h with 0.25 mCi/ml [^{32}P]orthophosphate, the tagged topo I was isolated on S protein-agarose beads. Samples were subjected to SDS-PAGE and analyzed by autoradiography. *C*, 3.0×10^7 K562 cells transfected with a plasmid encoding S-topo I (Tyr^{723}) were treated with 100 nM paclitaxel or diluent for 16 h followed by 0.25 mCi/ml [^{32}P]orthophosphate for 4 h. Topo I was isolated on S protein-agarose beads, subjected to SDS-PAGE, and analyzed by autoradiography as well as blotting with anti-S peptide antibody (25). The panel contains lanes from a single film exposure. *Dashes* indicate removal of unrelated lanes. *D*, after metabolic labeling with [^{32}P]orthophosphate, topo I was isolated by pulldown from 5.0×10^7 paclitaxel-treated cells stably expressing topo I-S (Y723F) as described in *B*, subjected to SDS-PAGE, and stained with Coomassie Brilliant Blue. The excised topo I band was subjected to two-dimensional tryptic mapping and PhosphorImager analysis as described under "Experimental Procedures." The sample origin is indicated by the *circle*. *E*, the stable line described in *B* was also used to obtain unlabeled, purified topo I for mass spectrometry as shown by a representative Coomassie Blue-stained gel. Results in *B*, *C*, *D* and *E* are representative of five, five, nine, and five experiments, respectively.

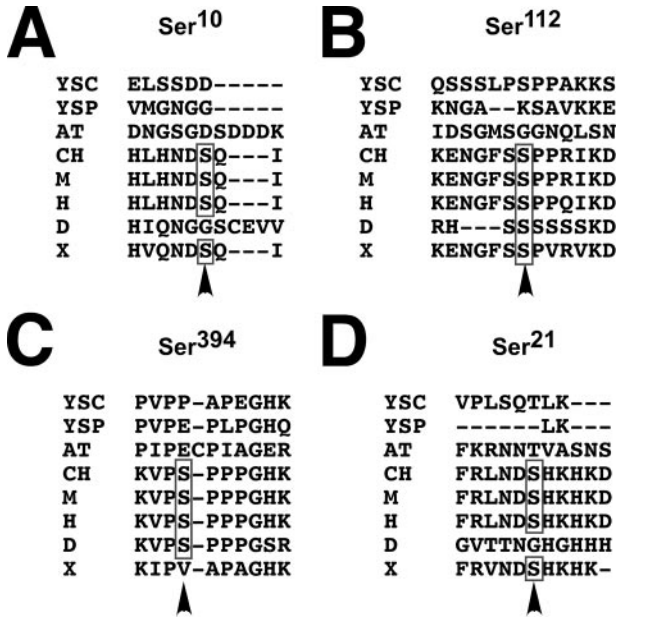

FIGURE 2. **Conservation of the four mapped topo I phosphorylation sites.** *A–D*, sequence homology of the four phosphorylation sites. *SC*, *Saccharomyces cerevisiae*; *SP*, *Schizosaccharomyces pombe*; *AT*, *Arabidopsis thaliana*; *X*, *Xenopus*; *M*, mouse; *CH*, chicken; *H*, human; D, *Drosophila melanogaster*.

with overexpressing active topo I in intact cells (4, 31), a plasmid encoding Topo I-S with an inactivating Y723F mutation was stably transfected into K562 cells. When the stable line was radiolabeled with [^{32}P]orthophosphate and the tagged topo I was pulled down using S protein-agarose beads, autoradiography showed that the tagged construct also incorporated ^{32}P (Fig. 1*B*). Consistent with an earlier report that murine topo I is differentially phosphorylated during mitosis (22), phosphorylation of Topo I-S (Y723F) increased in mitotic cells (Fig. 1*C*). Two-dimensional tryptic mapping suggested the possibility of four phosphorylated peptides in these mitotic cells (Fig. 1*D*).

Identification of Four Novel Phosphorylation Sites on Human Topo I—To identify topo I phosphorylation sites, unlabeled Topo I-S (Y723F) was isolated from stably transfected K562 cells and purified by SDS-PAGE. This procedure yielded a band of purified polypeptide that was easily visible after Coomassie Blue staining (Fig. 1*E*). Several samples prepared in this manner, including samples from untreated cells, cells arrested in mitosis by an 18-h treatment with 100 nM paclitaxel, and cells treated for 15 min with 50 nM phorbol myristate acetate, were subjected to trypsin digestion followed by quadrupole time-of-flight tandem mass spectrometry analysis. Results obtained with each of the three samples suggested that Ser^{10}, Ser^{112}, and Ser^{394} of topo I are phosphorylated in cells (supplemental Fig. S1, A–C).

Further analysis of the mass spectrometry data indicated that peptides corresponding to 20–30% of the polypeptide, especially the lysine-rich N-terminal domain, had not been detected. To maximize coverage of the polypeptide, additional samples were digested with the protease Arg-C instead of trypsin and subjected to mass spectrometry. Results of this analysis suggested that topo I is also phosphorylated at Ser^{21} (supplemental Fig. S1D).

The region around the phosphorylation sites was analyzed for sequence conservation. All four putative phosphorylation sites are conserved in higher eukaryotes, including mouse, chicken, and human, but not in yeast (Fig. 2, *A–D*). These findings suggest that these phosphorylations may be important for some function of the higher eukaryotic forms of topo I.

Ser^{10}, Ser^{21}, Ser^{112}, and Ser^{394} Are Phosphorylated during Mitosis—Phosphoepitope-specific anti-topo I antisera were generated to assess whether these four sites are phosphorylated on the endogenous topo I polypeptide in cells and to enable further analysis of the phosphorylation events. After affinity purification, each antiserum selectively detected phosphorylated polypeptide (Fig. 3*A*). Each phosphoepitope-specific antiserum detected topo I in K562 lysates by immunoblotting (Fig. 3*B*), indicating the endogenous phosphorylation at these sites in cells. Competition experiments (Fig. 3*C*) demonstrated that each affinity-purified reagent was specific for only its phosphoepitope.

To determine the phosphorylation state at these sites during various phases of the cell cycle, K562 cells were arrested at various stages of the cell cycle as verified by flow cytometry (supplemental Fig. S2) using aphidicolin (G_1/S), hydroxyurea (G_1/S), etoposide (G_2), and paclitaxel or nocodazole (M). Immunoblotting demonstrated that phosphorylation of Ser^{10},

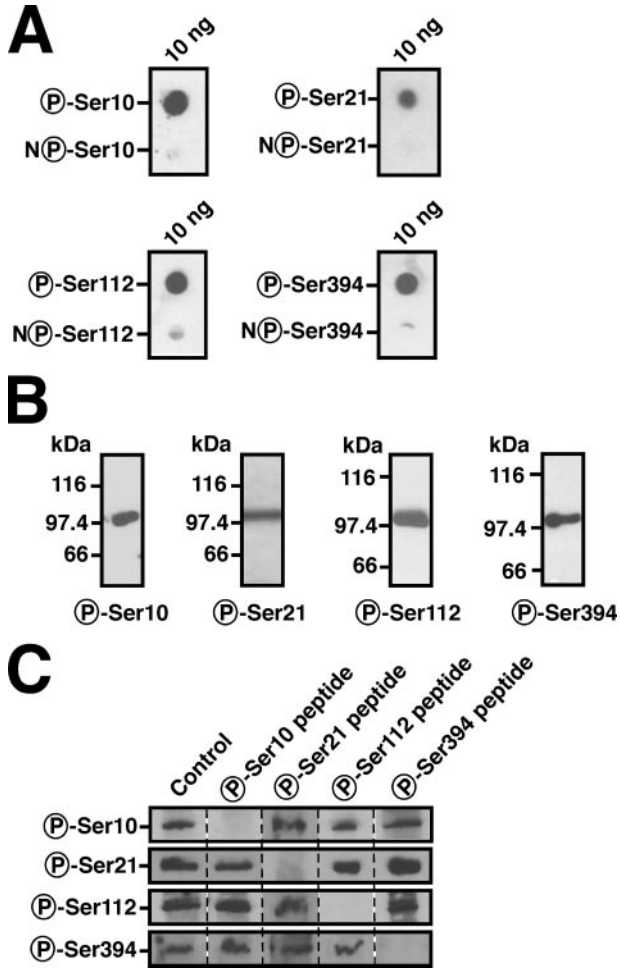

FIGURE 3. **Development of phosphotopo I antibodies.** *A* and *B*, polyclonal phosphoepitope-specific antibodies for Ser[10], Ser[21], Ser[112], and Ser[394] were generated in rabbits. Bleeds were affinity-purified as described under "Experimental Procedures" and screened by dot blots using phosphorylated (*P*) and nonphosphorylated (*NP*) peptides coupled to bovine serum albumin (*A*) and immunoblotting using K562 lysates (*B*). *C*, specificity of the anti-phosphoepitope antibodies. Affinity-purified antibodies were incubated with a 1 μg/ml concentration of the designated phosphopeptide during incubation with nitrocellulose-immobilized mitotic K562 lysates. Each panel contains lanes from a single film exposure. *Dashes* indicate removal of intervening lanes.

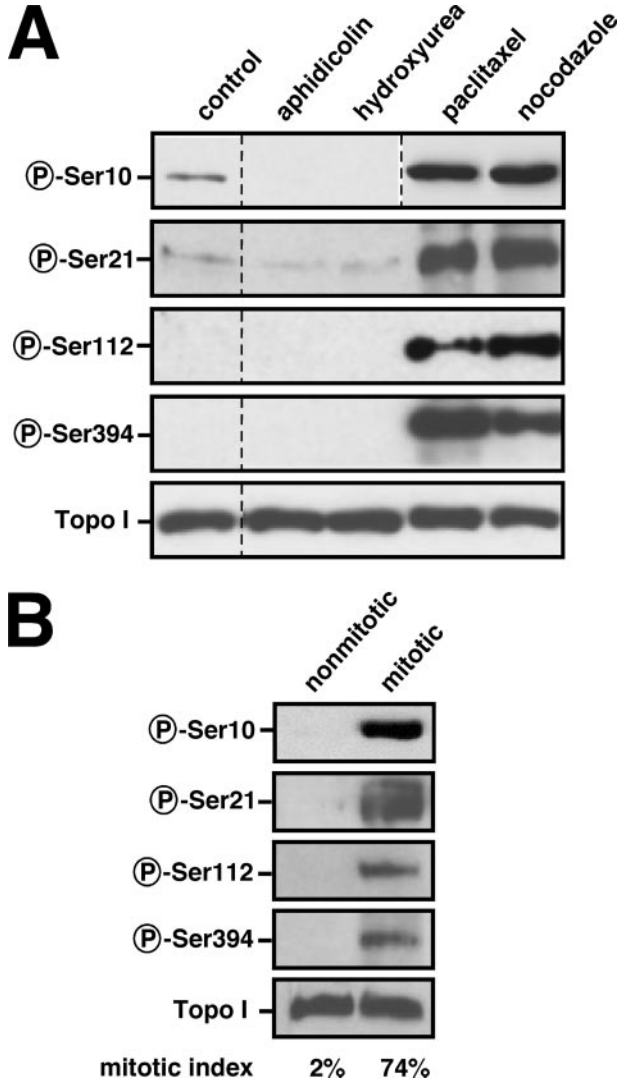

FIGURE 4. **Ser[10], Ser[21], Ser[112], and Ser[394] are phosphorylated during mitosis.** *A*, K562 cells were treated for 16 h with a variety of drugs, including 10 μM aphidicolin, 2 mM hydroxyurea, 100 nM paclitaxel, or 150 nM nocodazole, to cause arrest in various phases of the cell cycle. Changes in cell cycle distribution were confirmed by flow cytometry (supplemental Fig. S2). Samples were then subjected to SDS-PAGE and immunoblotting using anti-phospho-topo I antibodies. Each panel contains lanes from a single film exposure. *Dashes* indicate removal of lanes from cells subjected to additional treatments that did not affect Ser[10] phosphorylation. *B*, mitotic A549 cells were isolated by shake-off without drug treatment as described under "Experimental Procedures." Interphase cells were isolated at the completion of the shake-off, and the mitotic index of both interphase and mitotic cells was determined by microscopic examination after Hoechst 33258 staining. Samples were subjected to SDS-PAGE and immunoblotting. Representative topo I loading control blots are shown for both panels. Results in both panels are representative of five separate experiments. *P*, phosphorylated.

Ser[21], Ser[112], and Ser[394] was markedly increased only in cells treated with paclitaxel or nocodazole, suggesting that these phosphorylations occur predominantly or exclusively during mitosis (Fig. 4*A* and data not shown). To confirm this finding and verify that the mitotic phosphorylation of these sites was not caused by the drug treatment, mitotic A549 human lung cancer cells were separated from nonmitotic cells by mitotic shake-off. Analysis of these cells demonstrated phosphorylation of topo I at Ser[10], Ser[21], Ser[112], and Ser[394] in mitotic cells but not interphase cells (Fig. 4*B*).

In Vitro Phosphorylation Analysis—Analysis of the sequence around the four phosphorylation sites (Fig. 2) indicated that Ser[10] and Ser[21] are part of possible CKII and PKC consensus sequences, respectively. In addition, Ser[112] and Ser[394] are serine-proline sites, which are potential Cdk phosphorylation sites. Accordingly CKII, PKCα, and Cdk1 were tested for their ability to phosphorylate the sites *in vitro*. Topo I-S was pulled down from stably transfected K562 cells, washed, incubated with purified kinases *in vitro*, subjected to SDS-PAGE, and analyzed by immunoblotting with the phosphoepitope-specific antibodies. Alternatively purified topo I was incubated with purified kinases *in vitro* and subjected to the same protocol described above. Results demonstrated that Ser[10] can be phosphorylated by CKII, Ser[21] can be phosphorylated by PKCα, and Ser[112] and Ser[394] can be phosphorylated by Cdk1 *in vitro* (Fig. 5). Further analysis verified that each of these sites was phosphorylated specifically by its

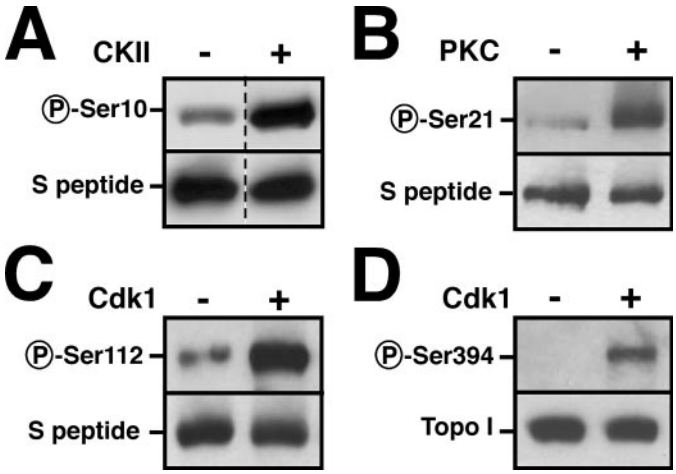

FIGURE 5. ***In vitro* kinase analysis.** Examination of the sequence around the phosphorylation sites (Fig. 2, *A–D*) tentatively identified kinases that could phosphorylate these sites. Topo I-S was pulled down from stably transfected K562 cells (Fig. 1*E*) and incubated with 200 μM ATP and 10 units of purified CKII, 10 units of Cdk1, or 10 ng of purified PKCα under conditions described under "Experimental Procedures." Alternatively purified topo I (Y723F) was incubated with 200 μM ATP and 10 units of Cdk1. At the completion of the incubation, samples were solubilized in SDS sample buffer and analyzed by immunoblotting using the anti-phospho-topo I antibodies. CKII was found to phosphorylate Ser^{10} (*A*), PKCα was found to phosphorylate Ser^{21} (*B*), and Cdk1 was found to phosphorylate Ser^{112} (*C*) and Ser^{394} (*D*) *in vitro*. Results are representative of five separate experiments. *P*, phosphorylated.

indicated kinase and not by the other two kinases (supplemental Fig. S3).

Phosphorylation Does Not Affect Topo I Localization or Interactions with Tested Binding Partners—To determine how phosphorylation of these sites affects topo I, site-directed mutagenesis was performed to create alanine mutants of S-Topo I. Because all four sites are phosphorylated during mitosis, a quadruple mutant (S10A/S21A/S112A/S394A, topo I 4A) construct was generated to remove all of these phosphorylation sites. Both catalytically active (Tyr^{723}) and inactive (Y723F) constructs were created.

Immunofluorescence was performed to monitor subcellular localization. After plasmids encoding wild type or 4A S-Topo I (Tyr^{723}) were transfected into K562 cells, localization of the polypeptide was analyzed using anti-S peptide antibody (Fig. 6*A*). 4A S-Topo I localized to the nucleus, including punctate regions representing nucleoli in interphase cells (supplemental Fig. S4A) and around the condensed chromosomes in mitotic cells (Fig. 6*A*, *lower panels*). These patterns are identical to the previously reported localization of endogenous topo I (36–39) and to the pattern seen with wild type S-Topo I in this study (Fig. 6*A*, *upper panels*). Similar results were also seen in paclitaxel-treated K562 cells (supplemental Fig. S4B). These results indicate that phosphorylation of these sites does not detectably affect topo I localization during mitosis.

To assess the effect of phosphorylation on the ability of topo I to interact with reported binding partners, a pulldown assay was performed. Following transfection with plasmids encoding either wild type or the 4A S-Topo I (Tyr^{723}), K562 cells were arrested in mitosis with paclitaxel. After isolation of topo I using S protein-agarose, samples were subjected to immunoblotting using antibodies against reported topo I binding partners, including nucleolin, poly(ADP-ribose) polymerase, TATA-binding protein, and topors (Fig. 6*B*). Wild type and 4A S-Topo I bound similar amounts of these polypeptides, indicating that phosphorylation of these four sites does not affect these protein-protein interactions.

Phosphorylation of Ser^{21} Stimulates Topo I Activity in Vitro and Enhances CPT-induced Cleavage Complex Stabilization in Cells—To assess the effect of phosphorylation on topo I activity, endogenous topo I extracted from untreated or paclitaxel-arrested K562 cells was assayed for enzymatic activity *in vitro*. In end point assays, serial 2-fold dilutions of the extracts were tested for the ability to relax a supercoiled plasmid during a 30-min incubation. Topo I isolated from mitotic cells exhibited 2–3-fold more relaxation activity than topo I isolated from untreated, interphase cells (Fig. 7*A*) as indicated by the dilution that resulted in reappearance of substrate (Fig. 7*A*, *graph*). Likewise time course experiments demonstrated that nuclear extracts from mitotic cells relaxed supercoiled substrate 2–4-fold more rapidly (Fig. 7*B*).

To verify that this change in activity is due to phosphorylation of topo I during mitosis, S-Topo I (Tyr^{723}) isolated from paclitaxel-arrested K562 cells was dephosphorylated with calf intestine alkaline phosphatase and assayed for topo I enzymatic activity using serial 2-fold dilutions of the pulldowns. Fractions of pulldowns treated with buffer or calf intestine alkaline phosphatase were set aside and analyzed by immunoblotting to verify equal expression (Fig. 8*A*, *inset*). Treatment with calf intestine alkaline phosphatase caused a 2-fold decrease in activity (Fig. 8*A*).

To determine whether this 2-fold decrease was related to dephosphorylation of one or more of the four phosphorylation sites mapped in this study, the activities of wild type and 4A S-Topo I (Tyr^{723}) isolated from paclitaxel-treated K562 cells were compared. The 4A mutant exhibited 2-fold lower levels of activity with equal expression of the two constructs (Fig. 8*B*). Conversely 4A and wild type S-Topo I exhibited similar levels of activity when 4A had higher levels of expression (Fig. 8*C*). Collectively these results indicate that one or more of the sites mapped in this study enhances topo I activity when phosphorylated.

To identify the phosphorylation(s) responsible for the altered activity, additional topo I mutants were created and analyzed. When isolated from mitotic cells, the 3A mutant (S10A/S112A/S394A) and wild type S-Topo I exhibited similar levels of activity (Fig. 8*D*), whereas the S21A mutant exhibited a 2-fold decrease in activity relative to wild type topo I (Fig. 8*E*). Importantly this difference in activity was not observed when wild type and S21A were pulled down from interphase cells (Fig. 8*F*), which lack Ser^{21} phosphorylation (Fig. 4*B*). Therefore, the decrease in relaxation activity seen in the 4A mutant *versus* wild type (Fig. 8*B*) and the wild type S-Topo I treated with calf intestine alkaline phosphatase (Fig. 8*A*) after isolation from mitotic cells is likely due to the absence or dephosphorylation, respectively, of Ser^{21}. These results indicate that phosphorylation of Ser^{21} during mitosis stimulates the DNA relaxation activity of topo I 2-fold *in vitro*.

To assess the possibility that the increased plasmid relaxation observed after Ser^{21} phosphorylation reflects increased DNA cleavage activity of the enzyme, the cleavage half-reaction was assayed using the strategy of Svejstrup *et al.* (40). In brief,

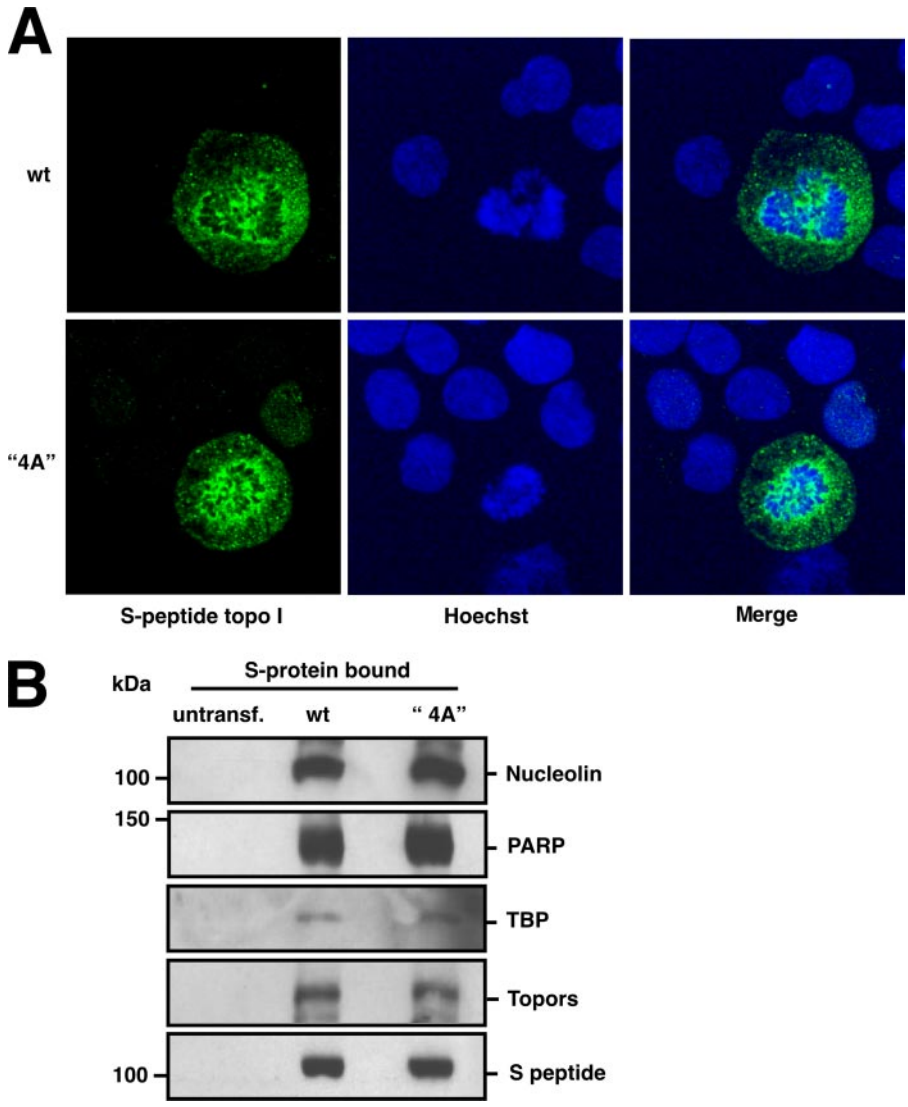

FIGURE 6. **Mutation of the four sites does not detectably affect localization or the assayed protein-protein interactions of topo I.** *A*, 24 h after transfection with plasmids encoding 4A or wild type (*wt*) S-topo I, log phase K562 cells were fixed with methanol and stained with anti-S peptide antibody followed by fluorescein-conjugated anti-mouse IgG and Hoechst 33258. Representative images of mitotic cells are shown. Note that the mitotic index is low because no spindle poisons were added. *B*, 6 h after transfection with plasmids encoding wild type (*wt*) or 4A S-topo I, K562 cells were treated for 16 h with 100 nM paclitaxel to induce mitotic arrest. Tagged S-topo I was isolated using S protein-agarose, subjected to SDS-PAGE, and analyzed by immunoblotting using antibodies that recognize known binding partners of topo I. Untransfected (*untransf.*) K562 cells were included as a negative control. Results are representative of three (*A*) or four (*B*) separate experiments. *PARP*, poly(ADP-ribose) polymerase; *TBP*, TATA-binding protein.

S-Topo I pulled down from mitotic cells was incubated with radiolabeled suicide cleavage substrate as indicated in Fig. 8*G*. With the amounts of topo I recovered after transient transfection, progressive cleavage of the substrate was observed over 5–45 min. Importantly 2-fold larger amounts of S21A topo I were required to yield the same cleavage rate as wild type topo I, suggesting that Ser^{21} phosphorylation is enhancing the rate of DNA cleavage.

To determine whether the *in vitro* changes in topo I activity correspond to an alteration in the behavior of topo I in intact cells, band depletion assays were performed to assess CPT-induced stabilization of covalent topo I-DNA complexes (34). K562 cells transiently transfected with plasmids encoding wild type or S21A S-Topo I were treated with paclitaxel for 16 h to allow mitotic phosphorylation and then exposed briefly to varying CPT concentrations in the continued presence of paclitaxel. The S21A mutant required higher CPT concentrations than wild type S-Topo I to trap the same amount of topo I in covalent topo I-DNA complexes (Fig. 9*A*). In contrast, CPT stabilized similar levels of cleavage complexes involving the two constructs when K562 cells were not arrested in mitosis (Fig. 9*B*). Consistent with these results, comparison of diluent- *versus* paclitaxel-treated cells revealed that stabilization of topo I-DNA complexes required lower CPT concentrations in mitotic than in log phase cells (Fig. 9*C*). These results indicate that Ser^{21} phosphorylation concomitantly enhances topo I activity (Fig. 8) and CPT sensitivity (Fig. 9) specifically during mitosis.

DISCUSSION

Results of the present study identified four phosphorylation sites on human topo I: Ser^{10}, Ser^{21}, Ser^{112}, and Ser^{394}. Further experiments demonstrated that all four sites are phosphorylated exclusively during mitosis. CKII and PKCα phosphorylated Ser^{10} and Ser^{21}, respectively, and Cdk1 phosphorylated Ser^{112} and Ser^{394} *in vitro*. Mutation of all four sites to alanine did not alter the mitotic localization or the assayed protein-protein interactions of topo I. Comprehensive mass spectrometric analyses also demonstrated that wild type and the 4A S-topo I mutant have similar binding partners.[3] However, both wild type topo I dephosphorylated by calf intestine alkaline phosphatase and the 4A mutant exhibited a 2-fold reduction in DNA relaxation activity *in vitro* relative to wild type topo I isolated from mitotic cells. Additional analysis determined that Ser^{21} phosphorylation is responsible for this change in enzymatic activity. Collectively these results suggest that phosphorylation plays a role in regulating topo I enzymatic activity and interaction with DNA during mitosis.

Mass spectrometry identified Ser^{10}, Ser^{21}, Ser^{112}, and Ser^{394} as four sites of topo I phosphorylation in intact cells (Fig. 2 and supplemental Fig. S1). Although phosphorylation of Ser^{10} had been suggested previously based on the ability of CKII to phos-

[3] J. S. Hackbarth and S. H. Kaufmann, unpublished observations.

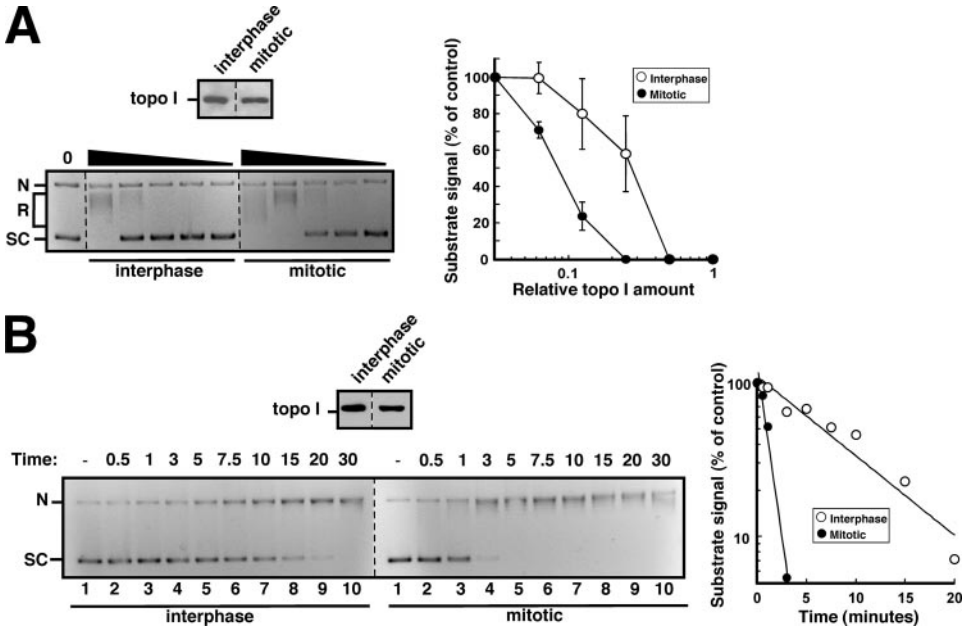

FIGURE 7. **DNA relaxation activity of wild type topo I from interphase and mitotic cells.** *A*, endogenous topo I was extracted from K562 cells treated with 0.1% DMSO or 100 nM paclitaxel to induce mitotic arrest as described under "Experimental Procedures." Serial 2-fold dilutions of the extracts were subjected to immunoblotting for topo I or used in a DNA relaxation assay to examine conversion from supercoiled plasmid (*SC*) to relaxed forms (*R*). Aliquots of the most concentrated extracts (*first lanes after dashed lines*) contained equal amounts of topo I (*inset*). The "*0*" *lane* contains plasmid that was incubated without extract. *Dashed lines* indicate removal of intervening lanes that contained unequal amounts of topo I. The *graph* shows substrate remaining at the end of the 30-min incubation. *Error bars*, mean ± S.E. of three independent experiments. *B*, extracts adjusted to contain equal amounts of topo I polypeptide (*inset*) from interphase or paclitaxel-arrested K562 cells were assayed for plasmid relaxation activity over time (*lanes 2–10*). *Lane 1*, untreated substrate. The *dashed line* indicates juxtaposition of two separate agarose gels from the same assay. The *graph* shows substrate remaining on the gel at each time point and the first order regression *line*. Results are representative of assays using three independently derived extracts. *N*, location of nicked and, in some assays, relaxed plasmid.

phorylate a small peptide corresponding to the N terminus of topo I (23), the other three sites had not been identified previously. All sites identified in the present study were located on serines in agreement with previous phosphoamino acid analysis (13, 18, 21). At least two previous studies, however, suggested that topo I can also be phosphorylated on tyrosine residues. Tse-Dinh *et al.* (41) found that tyrosine phosphorylation of topo I *in vitro* can alter its enzymatic activity. More recently, Yu *et al.* (24) reported that c-Abl can phosphorylate topo I on Tyr^{268} *in vitro*. Our mass spectrometry analysis did not detect phosphorylation of Tyr^{268}, although it is conceivable that the phosphorylated peptide was present in such low abundance that it could not be detected.

In further experiments we examined the kinases that can phosphorylate topo I on the sites we identified. Data base searches indicated that Ser^{10} and Ser^{21} conform to consensus phosphorylation sites for CKII and PKC, respectively. *In vitro* kinase assays (Fig. 5) verified this prediction, confirming and extending previous reports that CKII and PKC can phosphorylate topo I (16, 17). In addition, sequence analysis indicated that Ser^{112} and Ser^{394} are part of Ser-Pro sequences, which can be phosphorylated by Cdks or the mitogen-activated protein kinase family. Both sites were phosphorylated by Cdk1 *in vitro*, providing the first indication of a possible interaction between topo I and Cdk1. Cdk1 is active only during mitosis, which correlates with the finding that Ser^{112} and Ser^{394} are predominantly or exclusively phosphorylated during that phase of the cell cycle. Confirmation that each of these kinases is responsible for phosphorylating the respective sites in cells is not possible with the present technology. $CKII^{-/-}$ or $Cdk1^{-/-}$ cell lines do not exist due to the importance of these kinases for cell cycle progression. Moreover cells treated with inhibitors or small interfering RNA targeting these kinases are unable to enter mitosis. Thus, the identification of the kinases responsible for phosphorylating topo I must be considered tentative until new tools become available.

Experiments using phosphospecific topo I antibodies demonstrated that phosphorylation of all four sites was markedly increased during mitosis. These findings were observed both in cells treated with spindle poisons such as nocodazole and paclitaxel (Fig. 4*A*) and in untreated mitotic cells isolated by shake-off (Fig. 4*B*). These observations demonstrated that human topo I is phosphorylated in a cell-cycle dependent manner consistent with previous findings indicating that the murine topo I has a mitosis-specific phosphorylated form (22). Additional experiments that examined whether any of these sites are phosphorylated after various types of DNA damage (not shown) failed to demonstrate phosphorylation of these four sites after treatment with CPT or the topo II poison etoposide. In contrast, Ser^{10} was phosphorylated after treatment with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine or ionizing radiation consistent with the ability of DNA damage to activate CKII (42, 43).

Phosphorylation was reported previously to alter topo I enzymatic activity *in vitro*. Specifically dephosphorylation reportedly decreased or abolished topo I activity (13–15), and subsequent treatment with CKII or PKCα stimulated activity (14, 15). On the other hand, recombinant topo I, which has no detectable phosphorylation, was observed to be enzymatically active (44–46), casting doubt on the prior claim that phosphorylation is required for activity. Results of the present study were consistent with both sets of findings. After isolation from mitotic cells, dephosphorylation of topo I by calf intestine alkaline phosphatase caused a 2-fold decrease in plasmid relaxation activity (Fig. 8*A*). Because of the nonlinearity of the topo I relaxation assays, this result could potentially be interpreted as a more dramatic decrease if serial dilutions are not examined, possibly explaining earlier claims of more extensive activity loss upon dephosphorylation.

In further experiments, the 4A mutant exhibited the same 2-fold decrease relative to wild type enzyme after isolation from mitotic cells (Fig. 8*B* and *C*), indicating that one or more of the four mapped sites contributes to activity when phosphorylated during mitosis. Further analysis determined that Ser^{21} is the

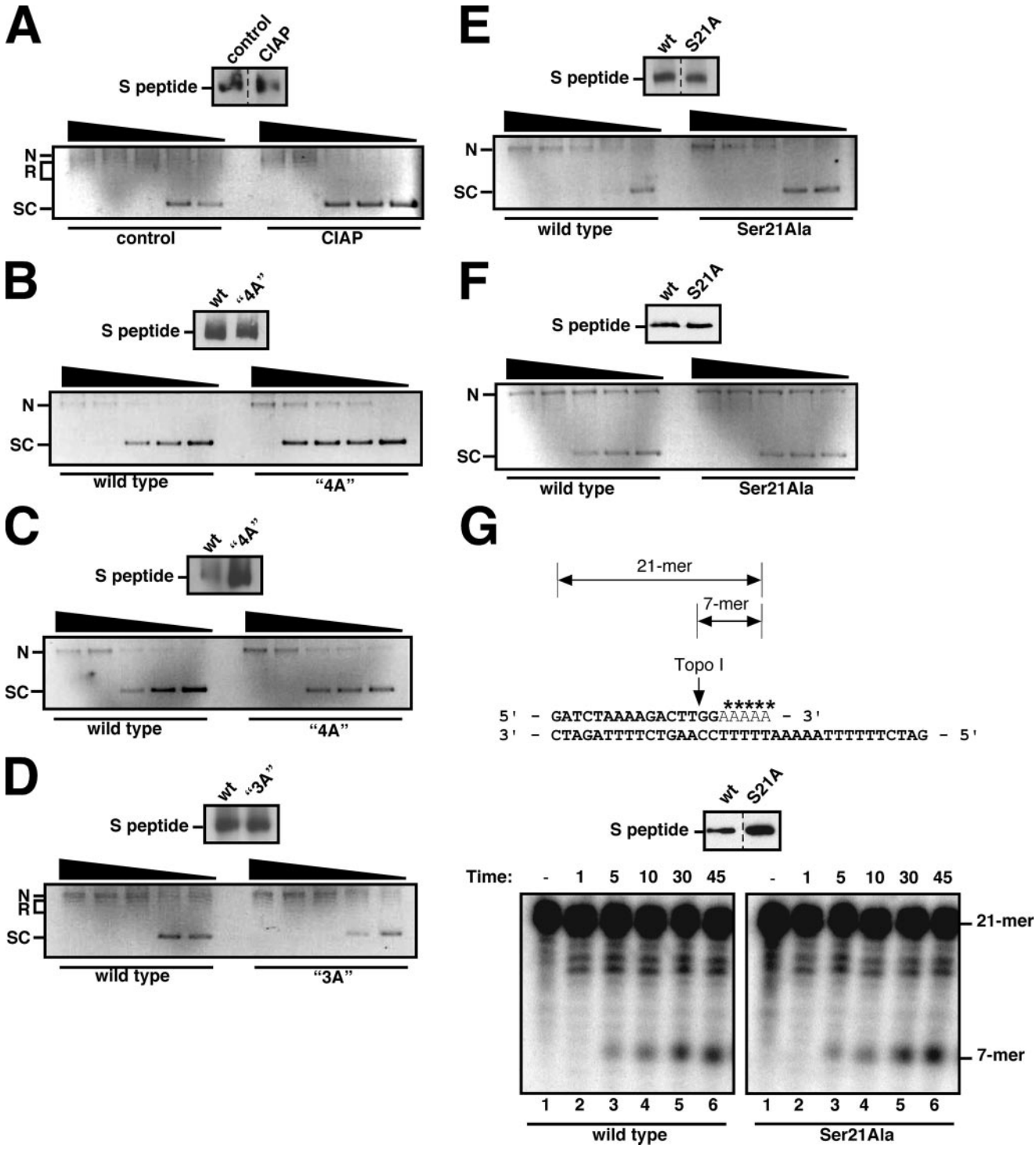

FIGURE 8. **Effect of Ser^{21} phosphorylation on topo I activity.** *A*, K562 cells were transiently transfected with wild type (*wt*) S-topo I (Tyr^{723}) and arrested in mitosis with 100 nM paclitaxel. S protein-agarose precipitates were treated with buffer or 10 units of calf intestine alkaline phosphatase (*CIAP*) as described under "Experimental Procedures." The beads were subjected to 2-fold dilutions in topo I assay buffer and used in a DNA relaxation assay. Aliquots of the immobilized topo I were also subjected to immunoblotting with anti-S peptide antibody to verify equal loading (see *insets*). The *inset* contains lanes from a single film exposure. *B–E*, wild type and 4A S-Topo I (*B* and *C*), wild type and 3A S-Topo I (*D*), or wild type and S21A S-Topo I (*E*) were isolated from paclitaxel-treated K562 cells and assayed as described in *A*. *F*, wild type and S21A S-Topo I were isolated from interphase K562 cells and assayed as described in *A*. Because of variability in transfection efficiency, experiments shown in *A–F* were conducted separately and cannot be directly compared. *G*, wild type and S21A S-Topo I isolated from paclitaxel-treated K562 cells were incubated with radiolabeled suicide substrate (*inset*) for the indicated length of time. *, radiolabeled nucleotide in substrate. At the completion of the reaction, the 21-mer substrate and 7-mer product were separated and visualized by phosphorimaging. Two separate gels from a single assay were imaged simultaneously. *Inset*, S peptide blot showing corresponding topo I contents of the pulldowns. *Dashes* indicate removal of extraneous lanes. Results are representative of three (*A*), 14 (*B* and *C*), four (*D*), three (*E*), five (*F*), and three (*G*) assays. *SC*, supercoiled; *R*, relaxed; *N*, location of nicked and, in some assays, relaxed plasmid.

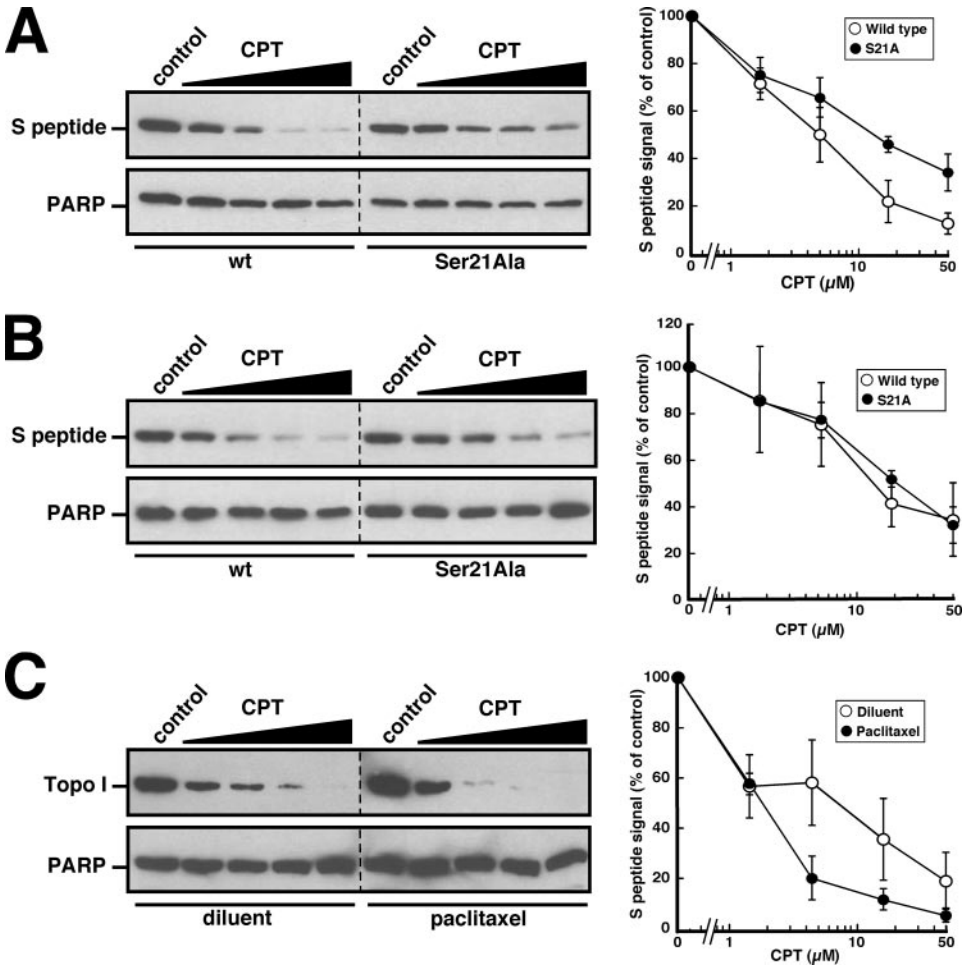

FIGURE 9. **The S21A topo I mutant is less sensitive to CPT-induced trapping on DNA in intact mitotic cells.** K562 cells transfected with plasmids encoding wild type (*wt*) or S21A S-topo I (Tyr^{723}) were treated with 100 nM paclitaxel for 16 h (*A*) or left untreated (*B*) before subjecting cells to a band depletion assay to assess CPT-induced stabilization of topo I-DNA complexes as described under "Experimental Procedures." The blots were probed with anti-S peptide antibody to specifically detect transfected constructs. *C*, K562 cells were treated with 100 nM paclitaxel or diluent for 16 h and then subjected to band depletion assay. Samples were subjected to SDS-PAGE, immunoblotting with anti-topo I to detect endogenous polypeptide, and densitometry using ImageJ. *Dashes* indicate removal of extraneous lanes. *Error bars*, mean ± S.E. of three (*A*), three (*B*), and five (*C*) experiments, respectively. *PARP*, poly(ADP-ribose) polymerase.

site that enhances activity when phosphorylated. A Ser21Ala mutant exhibited decreased ability to relax supercoiled plasmid (Fig. 8*E*) or cleave suicide substrate (Fig. 8*G*) *in vitro* as well as decreased CPT-induced cleavage complexes in mitotic cells (Fig. 9*A*) compared with wild type topo I. Importantly, these differences were not observed in interphase cells (Figs. 8*F* and 9*B*), where Ser^{21} is not phosphorylated (Fig. 4*B*).

Additional observations suggest that the effect of Ser^{21} phosphorylation is not limited to cells transfected with epitope-tagged topo I. Extracts from untransfected cells exhibited an increase in topo I activity during mitosis (Fig. 7, *A* and *B*) and enhanced sensitivity to CPT-induced stabilization of cleavage complexes (Fig. 9*C*) compared with interphase cells. Although the ability of phosphorylation in the N-terminal domain to affect events at the topo I active site might seem counterintuitive, previous studies have demonstrated that removal of the N-terminal domain modestly decreases topo I enzymatic activity and CPT sensitivity (47, 48). Crystallization of topo I with an intact N terminus, a feat that has not been reported to date, appears to be required to determine whether phosphorylation of Ser^{21} enhances activity and CPT sensitivity through an interaction with portions of the N-terminal domain previously implicated in topo I activation or through interaction with different regions of the polypeptide.

The results presented in this study reveal interesting similarities between the phosphorylation of topo I and topo II (49, 50), a structurally and mechanistically unrelated member of the topoisomerase family. Topo II is phosphorylated at numerous serine and threonine sites in cells and is phosphorylated by CKII, PKC, and Cdk1 *in vitro*. In addition, topo II has sites that are phosphorylated specifically during mitosis (51), and topo II activity peaks during mitosis (52, 53), presumably to facilitate chromosome separation. Although numerous studies have examined whether there is a link between topo II phosphorylation and its enzymatic activity, the results are conflicting especially when compared between species (54–58). The present study demonstrates that topo I activity likewise increases during mitosis, indicating that topo I might also participate in some way during that phase of the cell cycle.

In summary, the present study located and examined the four sites of endogenous phosphorylation on human topo I. All of these sites were phosphorylated predominantly during mitosis, demonstrating that topo I is phosphorylated in a cell cycle-dependent manner. Two of these sites, Ser^{112} and Ser^{394}, were phosphorylated *in vitro* by Cdk1, providing the first indication that a Cdk can modify topo I. Although the phosphorylation of these four sites did not detectably alter topo I localization or protein-protein interactions during mitosis, Ser^{21} phosphorylation enhanced topo I relaxation activity *in vitro* and CPT-induced stabilization of cleavage complexes in cells. These observations provide new understanding of the circumstances and effect of topo I phosphorylation in cells.

Acknowledgments—We gratefully acknowledge gifts of the topo I plasmid from M.-A. Bjornsti; antibodies from Guy Poirier, Naomi Rothfield, and Yung-Chi Cheng; assistance and analysis from Ross Tomaino and the Taplin Biological Mass Spectrometry Facility as well as the Flow Cytometry and Optical Morphology Shared Resource and the Peptide Synthesis Core at the Mayo Clinic; technical advice from L. James Maher and Robert McDonald; and editorial assistance from Deb Strauss.

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